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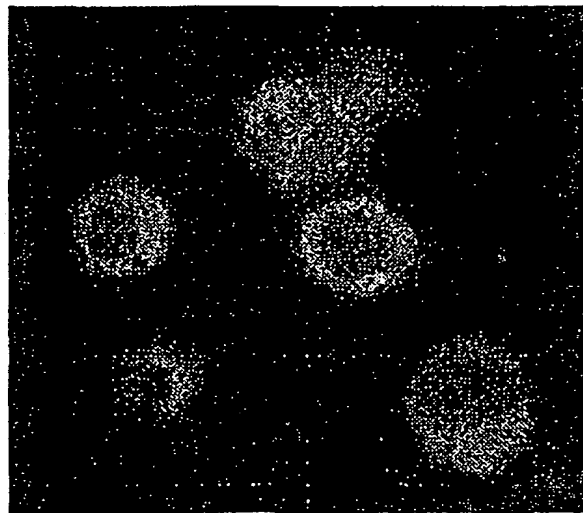
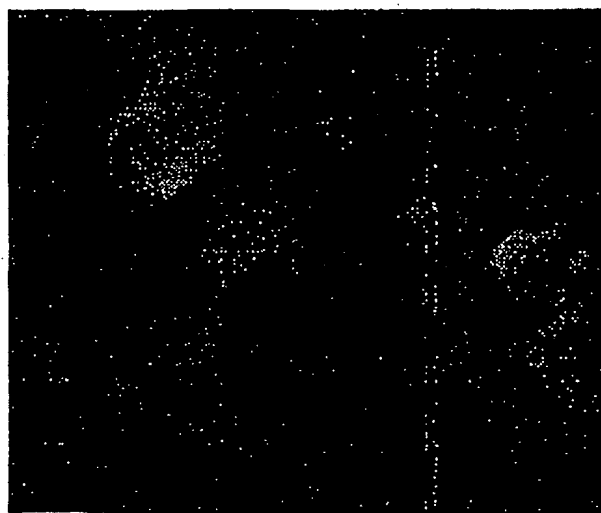
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(54) Title: LIPOSOMAL PHOSPHODIESTER, PHOSPHOROTHIOATE, AND p-ETHOXY OLIGONUCLEOTIDES



## (57) Abstract

An improved delivery system for antisense oligonucleotides involves a liposomal composition, comprising a liposome which consists essentially of neutral phospholipids and an antisense oligonucleotide that is entrapped in the liposome and is selected from the group consisting of phosphodiester oligonucleotides, phosphorothioate oligonucleotides, and p-ethoxy oligonucleotides.

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10           LIPOSOMAL PHOSPHODIESTER, PHOSPHOROTHIOATE, AND  
              p-ETHOXY OLIGONUCLEOTIDES

15           The present invention relates to liposomal formulations of certain antisense  
oligonucleotides, specifically liposomal phosphodiester, phosphorothioate, and p-  
ethoxy oligonucleotides. The invention also relates to methods of making such  
formulations and methods of using such formulations in medical therapy.

20           Antisense oligonucleotides (oligos), complementary to specific regions of the  
target mRNA, have been used to inhibit the expression of endogenous genes. When  
the antisense oligonucleotides bind to the target mRNA, a DNA-RNA hybrid is  
formed. This hybrid formation inhibits the translation of the mRNA and, thus, the  
gene's expression of the protein. If the protein is essential for the survival of the cell,  
the inhibition of its expression may lead to cell death. Therefore, antisense  
oligonucleotides can be useful tools in anticancer and antiviral therapies.

25           The main obstacles in using antisense oligonucleotides to inhibit gene  
expression are cellular instability, low cellular uptake, and poor intracellular delivery.  
Natural phosphodiesters are not resistant to nuclease hydrolysis; thus high  
concentrations of antisense oligonucleotides are needed before any inhibitory effect is  
observed. Modified phosphodiester analogs, such as phosphorothioates, have been

made to overcome this nuclease hydrolysis problem, but they have not provided a completely satisfactory solution to the problem.

The cellular uptake of antisense oligonucleotides is low. To solve this problem, physical techniques such as calcium-phosphate precipitation, DEAE-dextran  
5 mediation, or electroporation have been used to increase the cellular uptake of oligonucleotides. These techniques are difficult to reproduce and are inapplicable in vivo. Cationic lipids, such as Lipofectin, have also been used to deliver phosphodiester or phosphorothioate oligonucleotides. An electrostatic interaction is formed between the cationic lipids and the negatively charged phosphodiester or  
10 phosphorothioate oligonucleotides, which results in a complex that is then taken up by the target cells. Since these cationic lipids do not protect the oligonucleotides from nuclease digestion, they are only useful in delivering the nuclease-resistant phosphorothioates, but not the nuclease-cleavable phosphodiesters.

Another modified phosphodiester (PD) analog that has been prepared is p-ethoxy (pE) oligos. The modifications of pE oligos are made in the phosphate  
15 backbone so that the modification will not interfere with the binding of these oligos to the target mRNA. pE oligos are made by adding an ethyl group to the nonbridging oxygen atom of the phosphate backbone, thus rendering these oligos uncharged compounds. In spite of their resistance to nucleases, the cellular uptake and  
20 intracellular delivery of pE oligos are still poor because upon internalization, these oligos remain sequestered inside the endosomal/lysosomal vacuoles, impeding their access to the target mRNA.

There is a need for improved antisense compositions for use in treatment of disease, and also a need for processes for making such improved compositions.

25 One embodiment of the present invention is a liposomal composition of antisense oligonucleotides. The composition includes (a) a liposome which consists essentially of neutral phospholipids, and (b) an antisense oligonucleotide that is

entrapped in the liposome and is selected from the group consisting of phosphodiester oligonucleotides, phosphorothioate oligonucleotides, and p-ethoxy oligonucleotides. The phospholipids are preferably phosphatidylcholines. An especially preferred phospholipid is dioleoylphosphatidyl choline. When the antisense oligonucleotide is a phosphodiester oligonucleotide, the preferred molar ratio of phospholipid to oligo is less than about 3,000:1. When the antisense oligonucleotide is a phosphorothioate oligonucleotide, the preferred molar ratio of phospholipid to oligo is between about 10:1 and about 50:1. When the antisense oligonucleotide is a p-ethoxy oligonucleotide, the preferred molar ratio of phospholipid to oligo is between about 5:1 and about 100:1.

Another embodiment of the present invention is a method of inhibiting the growth of tumor cells in mammals. The method comprises the step of administering to a mammalian subject having a tumor an amount of the above-described composition that is effective to inhibit the growth of tumor cells.

Another embodiment of the present invention is a method of preparing the liposomal composition of antisense oligonucleotides. The method comprises the steps of (a) hydrating a lyophilized composition that consists essentially of neutral phospholipids and an antisense oligonucleotide that is selected from the group consisting of phosphodiester oligonucleotides, phosphorothioate oligonucleotides, and p-ethoxy oligonucleotides, thereby forming an aqueous suspension which includes free oligonucleotide and liposomes entrapping oligonucleotide; and (b) separating the free oligonucleotide from the liposomes by dialysis. In a preferred embodiment, the aqueous suspension is sonicated before dialysis.

The compositions of the present invention constitute an improved delivery system for antisense oligos, such as those used in anti-cancer therapy. In addition to minimizing nuclease hydrolysis of the oligos, the liposomal compositions of the present invention result in increased cellular uptake and intracellular delivery of the antisense oligos, as compared to prior art compositions. Therefore, when such

compositions are used to deliver oligos that inhibit the expression of a gene found in cancerous cells but not in normal cells, the therapeutic results are enhanced. Cancers which may be treated in accordance with the present invention would be numerous, with leukemias being one prominent example.

- 5           The formulations of the present invention also enhance the incorporation of oligos in the liposomes, as compared to prior art liposomal formulations.

Figures 1A and 1B are photographs showing the uptake of (A) free and (B) liposomal pE oligos by ALL-1 cells.

- 10           Figure 2A shows the extent of growth inhibition that resulted when ALL-1 and HL60 cells were incubated with liposomal-pE antisense oligos targeted against the B1/A2 breakpoint junction of Bcr-Abl mRNA.

Figure 2B shows the extent of growth inhibition that resulted when ALL-1 cells were incubated with B1/A2 liposomal-pE antisense oligos and B2/A2 liposomal-pE control oligos.

- 15           Figure 3A shows the extent of growth inhibition that resulted when BV173 and HL60 cells were incubated with liposomal-pE antisense oligos targeting against the B2/A2 breakpoint junction of Bcr-Abl mRNA.

- 20           Figure 3B shows the extent of growth inhibition that resulted when BV173 cells were incubated with liposomal-pE antisense oligos targeting against the B2/A2 breakpoint junction and with control oligos targeting against the B1/A2 breakpoint junction.

Figure 4A shows the extent of growth inhibition that resulted when K562 and HL60 cells were incubated with liposomal-pE antisense oligos targeting against the B3/A2 breakpoint junction of Bcr-Abl mRNA.

Figure 4B shows the extent of growth inhibition that resulted when K562 cells were incubated with liposomal-pE antisense oligos targeting against the B3/A2 breakpoint junction and with control oligos targeting against the B1/A2 breakpoint junction.

5           Figure 5 shows the extent of growth inhibition that resulted when Tween 20-containing liposomal-pE oligos were incubated with BV173 cells.

"Liposomes" is used in this patent to mean lipid-containing vesicles having a lipid bilayer, as well as other lipid carrier particles which can entrap antisense oligonucleotides. The liposomes can be made of one or more phospholipids, as long  
10 as the lipid material is substantially uncharged. It is important that the composition be substantially free of anionic and cationic phospholipids and cholesterol. Suitable phospholipids include phosphatidyl cholines and others that are well known to persons that are skilled in this field. The liposomes can be, for example, unilamellar, multilamellar, or have an undefined lamellar structure. A pharmaceutical composition  
15 comprising the liposomes will usually include a sterile, pharmaceutically acceptable carrier or diluent, such as water or saline solution.

"Entrap," "encapsulate," and "incorporate" are used in this patent to mean that the oligo is enclosed within at least some portion of the inner aqueous space (including the interlamellar regions of the bilayer) of the liposome.

20           A composition of the present invention is preferably administered to a patient parenterally, for example by intravenous, intraarterial, intramuscular, intralymphatic, intraperitoneal, subcutaneous, intrapleural, or intrathecal injection, or may be used in ex vivo bone marrow purging. Preferred dosages are between 5-25 mg/kg. The administration is preferably repeated on a timed schedule until the cancer disappears  
25 or regresses, and may be in conjunction with other forms of therapy.

The making and use of the present invention is further illustrated by the following examples.

## EXAMPLE 1

### Materials

5           Phosphodiester and phosphorothioate oligonucleotides were provided by Genta Incorporated. Phospholipids were purchased from Avanti Polar Lipids.

### Oligonucleotide Labeling

Phosphodiesters were labeled at 37°C for 8 h with [<sup>32</sup>P<sub>γ</sub>]ATP at the 5' end by T4 kinase. The labeled oligonucleotide was precipitated with ethanol at -20°C  
10           overnight. After washing with 70% ethanol three times, phosphodiester oligonucleotides were twice filtered with a Microcon-3 filter to separate the labeled oligonucleotides from free [<sup>32</sup>P<sub>γ</sub>]ATP.

Phosphorothioates, labeled with <sup>35</sup>S, were provided by Genta Incorporated.

### Liposomal-phosphodiester Preparation

15           Phosphodiester oligonucleotides dissolved in distilled water were mixed with phospholipids in the presence of excess t-butanol so that the final volume of t-butanol in the mixture was 80-90%. Trace amounts of [<sup>3</sup>H]cholestanyl ether and [<sup>32</sup>P]phosphodiester were also added to the mixture as lipid and oligonucleotide markers, respectively. The mixture was vortexed before being frozen in an  
20           acetone/dry ice bath. The frozen mixture was lyophilized and hydrated with hepes buffered saline (1 mM Hepes and 10mM NaCl) overnight. Liposomes were twice sonicated for 10 min in a bath type sonicator.

### Liposomal-phosphorothioate Preparation

Liposomal-phosphorothioates were prepared similarly to that described for  
25           liposomal-phosphodiesters, except phosphorothioates, instead of phosphodiesters, were used. Also, [<sup>35</sup>S]phosphorothioates, instead of [<sup>32</sup>P]phosphodiesters, were used as the oligonucleotide marker.



### Separation of Free Oligonucleotides from those Incorporated in Liposomes

The separation of free phosphodiester or phosphorothioate oligonucleotides from those incorporated in liposomes was done by dialyzing the mixture against an excess 2500-fold volume of RPMI medium at room temperature overnight. Aliquots  
5 of the preparation were taken before and after dialysis for liquid scintillation counting to assess the incorporation of phosphodiester or phosphorothioate oligonucleotides in liposomes.

### Development of liposomal-phosphodiesters

Dioleoylphosphatidylcholine (DOPC) lipids were chosen for the  
10 phosphodiester (PD) incorporation because they are neutral while PD is negatively charged. By using this lipid, electrostatic repulsion (which can lower incorporation) may be reduced. Positively charged lipids were not used because they may induce non-specific cellular toxicity. The initial attempts of incorporating PD oligonucleotides into liposomes were done by using freeze-and-thaw, and  
15 dehydration-rehydration methods.

#### (A) Freeze-and-thaw (FT) Method.

[<sup>3</sup>H]labeled DOPC lipids were evaporated from their organic solvent under nitrogen gas to form a lipid film. After vacuum desiccation, the lipid film was hydrated with hepes buffered saline (1 mM Hepes, 10 mM NaCl, pH 8.0) and  
20 sonicated in a bath type sonicator. These pre-formed liposomes were then mixed with [<sup>32</sup>P]labeled PD oligonucleotides at a 100 or 1000 to 1 molar ratio. The whole mixture was frozen in an acetone/dry ice bath for 5-10 minutes and thawed at room temperature for 25-30 minutes. This process was repeated three times before the sample was loaded on a BioGel A0.5M column for separation of free PD  
25 oligonucleotides from liposomal-PD. Aliquots were taken before and after the freeze-thaw procedure and were sent to liquid scintillation counting to determine the incorporation of phosphodiesters into liposomes.

Incorporation was determined by

$$\frac{[^{32}\text{P}]\text{PD}/\mu\text{mole } [^3\text{H}]\text{DOPC after freeze-thaw}}{[^{32}\text{P}]\text{PD}/\mu\text{mole } [^3\text{H}]\text{DOPC before freeze-thaw}} \times 100\%$$

There was no incorporation (Table 1).

#### (B) Dehydration-Rehydration (DR) Method

5            $[^3\text{H}]$ labeled DOPC was evaporated from the organic solvent under nitrogen gas to form a lipid film. After vacuum desiccation, the lipid film was hydrated with hepes buffered saline and sonicated in a bath type sonicator. These pre-formed liposomes were then frozen in an acetone-dry ice bath and lyophilized. The dried lipids were then rehydrated with distilled water containing  $[^{32}\text{P}]$ phosphodiester oligonucleotides. The molar ratio of DOPC to PD was either 100/1 or 1000/1. The mixture was loaded on a BioGel A0.5M column for separation of free PD oligonucleotides from liposomal-PD. Aliquots were taken before and after column loading and were sent to liquid scintillation counting. The incorporation was <5% (Table 1).

15

Table 1

Comparison of Freeze-thaw (FT) and Dehydration-rehydration (DR) methods on the incorporation of PD into DOPC liposomes.

Molar ratio	Methods	% incorporation
100/1	FT	0
	DR	2.0
1000/1	FT	0
	DR	4.6

20           With 500/1 and 5000/1 molar ratios of DOPC to PD, 0 and 5.2% incorporation were obtained, respectively.

It was then found that by adding t-butanol to the mixture before freezing and lyophilization, incorporation was raised to 11.9%. Also, by reducing the volume of distilled water added during the rehydration process from 200 to 50  $\mu\text{l}$ , 11.9 versus

1.8% incorporation was obtained with 1000/1 molar ratio. Effect of molar ratio on the incorporation was again measured with these improved conditions (Table 2).

Table 2

Effect of lipid to oligonucleotides molar ratio on the incorporation of PD  
5 into liposomes.

Molar ratio	% incorporation
1000/1	11.9
2000/1	17.0
3000/1	13.4
4000/1	5.5
5000/1	9.2

PD dissolved in distilled water was mixed with DOPC in the presence of excess t-butanol so that the final volume of t-butanol in the mixture was 80-90% before being frozen in an acetone/dry ice bath and lyophilized. This was to avoid  
10 the step of preparing pre-formed liposomes. With this procedure, similar level of incorporation (16.2%) was obtained at the 2000/1 molar ratio.

It was decided to change the method of separation of free PD from liposomal-PD because the recovery of lipids and PD were < 50%. Two other separation methods were used: Microcon-10 filters and dialysis (Table 3).

15

Table 3

Comparison of different methods of separating free PD from PD incorporated in liposomes.<sup>a</sup>

Method	% Incorporation	% Lipid and PD recovery
Column	11.9	40-50
Filter	44.7	60-70
Dialysis	76.7	> 90

<sup>a</sup> DOPC was used to incorporate PD oligonucleotides at a 1000/1 molar ratio.

When the liposomal mixture was sonicated for 10 min before the dialysis separation process, similar level of incorporation was obtained. Another lipid, dimyristoyl phosphatidylcholine, was also used for PD incorporation. There was > 85% incorporation.

#### Development of liposomal-phosphorothioates

Similar incorporation protocol was used with phosphorothioates (PT) since PT and PD are structural analogs. Various molar ratios of DOPC to PT were used (Table 4). The effect of sonication of the liposomal mixture (before dialysis) was also studied.

Table 4

Effect of lipid to oligonucleotide molar ratios on the incorporation of PT into liposomes.

Molar ratio	% incorporation without sonication	with sonication
10/1	> 90	> 90
50/1	> 90	> 90
100/1	45.8	55.5
200/1	44.1	49.1
500/1	27.8	47.0
1000/1	25.1	42.1

#### 15 EXAMPLE 2

##### Incorporation of p-ethoxy oligos into liposomes

pE oligos were purchased from Oligos Therapeutics (Willsonville, OR). Phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

(a) Oligo labeling

pE oligos were labeled at 37°C for 24 h with [<sup>32</sup>P<sub>γ</sub>]ATP at the 5' end by T4 polynucleotide kinase, and then precipitated with ethanol at -20°C overnight. They were then washed with 70% ethanol three times to separate the labeled oligo from  
5 free [<sup>32</sup>P<sub>γ</sub>]ATP.

(b) Liposome preparation

pE oligos dissolved in distilled H<sub>2</sub>O were mixed with phospholipids at various molar ratios in the presence of excess t-butanol so that the final volume of t-butanol in the mixture was at least 95%. Trace amounts of [<sup>3</sup>H]cholestanyl ether  
10 and [<sup>32</sup>P]pEs were also added to the mixture as lipid and oligo markers, respectively. The mixture was vortexed, frozen in an acetone/dry ice bath and then lyophilized. The lyophilized preparation was hydrated with Hepes buffered saline (1 mM Hepes and 10 mM NaCl) at a final oligo concentration of 10-100 μM. The liposomal-p-ethoxy oligos were sonicated for 10-20 min in a bath type sonicator.

15 (c) Separation of free pE oligos from those incorporated in liposomes

The separation of free pE oligos from those incorporated in liposomes was done by dialyzing (MW cutoff = 12-14,000) against 1000-fold excess of Hepes buffered saline at room temperature overnight. Aliquots of liposomal-pE oligos were taken before and after dialysis for liquid scintillation counting to access the  
20 incorporation of pE oligos in liposomes.

(d) Incorporation efficiency

The lipid phosphatidylcholine (PC) was chosen for the incorporation of pE oligos because both PC and pE oligos are neutral molecules so they should be compatible. Among all the different PCS, dioleoyl PC (DOPC) was chosen because  
25 it has a chain-melting phase transition temperature at -15 to -20°C. Thus, at room temperature, DOPC is in the liquid crystalline phase which is the ideal phase to prepare liposomes.

To incorporate pE oligos into liposomes, different molar ratios of pE oligos were mixed with DOPC together in the presence of excess of t-butanol. Trace amounts of radio labeled pE oligos and DOPC were included in the mixture. The DOPC/pE oligos mixtures were frozen in a dry ice/acetone bath before being  
5 lyophilized. The lyophilized DOPC/pE oligo powder was then hydrated with Hepes buffered saline so that the final oligo concentration was 10  $\mu$ M. pE oligos were successfully incorporated into DOPC liposomes, ranging between 28 to 83% efficiency (Table 5). The incorporation efficiency was dependent on the molar ratios of DOPC to pE oligos: 10 > 100 > 5 > 1000:1.

Table 5

Effect of molar ratio of DOPC to pE oligos on the incorporation of pE oligos

Molar ratio of DOPC:pE oligos	Incorporation efficiency (%) <sup>a</sup>
5:1	45
10:1	83
100:1	71
1000:1	28

<sup>a</sup>The incorporation efficiency values were obtained from three separate experiments.

#### 5 Delivery of pE oligos to leukemic cells

After achieving a high incorporation efficiency (> 80%) of pE oligos into liposomes, we then proceeded to test whether these liposomal-pE oligos can reach the cytoplasm in which the mRNA is located. We had purchased a 16-mer pE oligo labeled with rhodamine at the 5' end so that we could visualize the localization of the pE oligos by fluorescent microscopy.

##### (a) Incubation of pE oligos with leukemic cells

ALL-1 cells, which are human acute lymphocytic leukemic cells, were used. Fifty thousand ALL-1 cells/well were plated in a 24-well plate in 0.3 mL of medium. After 2 h of plating, final concentrations of 16  $\mu$ M of liposomal or free pE oligos conjugated with rhodamine were added to ALL-1 cells. After 24 h of incubation, the cells were thrice washed with phosphate buffered saline before being viewed under a confocal laser scanning microscope. See Figure 1 (Uptake of (A) free or (B) liposomal-pE oligos by ALL-1 cells).

Our data indicates that when incorporated into liposomes, higher amounts of pE oligos were taken up by the ALL-1 cells. The liposomes were able to deliver the pE oligos to the cytoplasm.

Growth inhibition of liposomal-pE oligos on leukemic cells

We then proceeded to test whether liposomal-pE oligos can specifically inhibit the growth of leukemic cells. We have used three different kinds of human leukemic cell lines: ALL-1 (acute lymphocytic leukemia), BV173 and K562 (both are chronic myelogenous leukemia). All three cell lines contain the rearranged Philadelphia (Ph) chromosome which arises from a reciprocal translocation of chromosomes 9 and 22. This translocation results in the relocation of the c-Abl protooncogene from chromosome 9 onto the 3' end of the breakpoint cluster region (Bcr) of chromosome 22, thus producing a hybrid Bcr-Abl gene. The breakpoint junctions where the Bcr and the Abl genes fuse are different in the three cell lines. In ALL-1 cells, the breakpoint junction is Bcr exon 1/ Abl exon 2. In BV173 cells, the breakpoint junction is Bcr exon 2/ Abl exon 2. In K562 cells, the breakpoint junction is Bcr exon 3/ Abl exon 2. All these hybrid genes produce a novel Bcr-Abl fusion protein, which has enhanced tyrosine kinase activity that has been linked to the pathogenesis of the leukemias. Thus, inhibition of the production of the Bcr-Abl protein may lead to leukemic cell growth inhibition and even cell death. To specifically inhibit the production of the Bcr-Abl protein, we have decided to target the antisense sequences against the breakpoint junctions of the Bcr-Abl mRNA which is only found in Ph chromosome-positive leukemic cells, but not normal cells. This way, we hope to induce minimal non-specific toxic side effects as only leukemic, not normal, cell growth will be affected.

(a) Sequences of the pE antisense oligos (written from 5' to 3' end)

antisense against Bcr exon 1/ Abl exon 2 (B1/A2) found in ALL-1 cells

GAAGGGCTTCTGCGTC

antisense against Bcr exon 2/ Abl exon 2 (B2/A2) found in BV173 cells

CTGAAGGGCTTCTTCC

antisense against Bcr exon 3/ Abl exon 2 (B3/A2) found in K562 cells

GGGCTTTTGA ACTCTGCT



(b) Delivery of liposomal-pE oligos to leukemic cells

Ten thousand ALL-1 or BV173 cells or five thousand K562 cells were plated per well in a 96-well plate in 100  $\mu$ L of RPMI medium containing 10% fetal calf serum. After 2 h of plating, final concentrations of 0 - 10  $\mu$ M of liposomal-pE oligos were added to leukemic cells. The cells were incubated with liposomal-pE oligos for 5 days. HL60 cells, a human promyelocytic cell line which does not have the Philadelphia chromosome, were used as control cells. They were plated under the same conditions at ten thousand cells/well.

(c) Determination of the viability of the leukemic cells

At the end of the incubation, 100  $\mu$ L of medium were added to each well which makes the final volume of each well to be 200  $\mu$ L. Then 50  $\mu$ L of cells were aliquoted and added to 96-well plates containing 130  $\mu$ L of medium and 20  $\mu$ L of alamarBlue dye. The cells will be incubated for 4-8 more hours at 37°C before being read directly on a microplate reader (Molecular Devices, Menlo Park, CA) at 570 and 595 nm. The alamarBlue dye incorporates an oxidation-reduction indicator that changes color in response to chemical reduction of growth medium resulting from cell growth. The difference in absorbance between 570 and 595 nm will be taken as the overall absorbance value of the leukemic cells. The viabilities of leukemic cells treated with liposomal-pE oligos will be compared with those of the control untreated cells.

When ALL-1 and HL60 cells were incubated with liposomal-pE antisense oligos targeted against the B1/A2 breakpoint junction of Bcr-Abl mRNA, a dose-dependent growth inhibition of ALL-1, but not HL60, cells was observed (Fig. 2A). Similarly growth inhibition was observed with BV173 and K562 cells when they were incubated with liposomal-pE antisense oligos targeting against the B2/A2 and B3/A2 breakpoint junctions of Bcr-Abl mRNA, respectively (Fig. 3A, 4A). Under identical conditions, HL60 cells were not growth-inhibited.

To ensure that the growth inhibitory effects were sequence-dependent, the Ph chromosome-positive cell lines were incubated with antisense and control liposomal-pE oligos. When ALL-1 cells were incubated with the B1/A2 liposomal-pE antisense oligos and the B2/A2 liposomal-pE control oligos, growth inhibition  
5 was induced (Fig. 2B). However, the B1/A2 antisense oligos induced a much greater inhibitory effect. Similarly, higher inhibitory effects on BV173 and K562 cells were found with the corresponding liposomal-pE antisense oligos than with the control oligos (Fig. 3B, 4B).

We also found that by including the detergent Tween 20 in the liposomal-pE  
10 oligo mixture, the potency of the inhibitory effects of the liposomal-pE oligos was increased. We added Tween 20 at 5% (wt. of pE oligos) in the liposomal-pE oligo mixture. Then the mixture was vortexed, frozen in an acetone/dry ice bath before being lyophilized. The dried mixture was then hydrated and sonicated as stated previously. When Tween 20-containing liposomal-pE oligos were added to BV173  
15 cells, 100% growth inhibition was observed at 5  $\mu$ M (Fig. 5) whereas under the same conditions, 100% growth inhibition with normal liposomal-pE oligos (no Tween 20) was observed at 10  $\mu$ M instead.

\* \* \*

The preceding description of specific embodiments of the present invention  
20 is not intended to be a complete list of every possible embodiment of the invention. Persons skilled in this field will recognize that modifications can be made to the specific embodiments described here that would be within the scope of the present invention.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Board of Regents, The  
University of Texas System
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PHOSPHOROTHIOATE, AND p-ETHOXY OLIGONUCLEOTIDES
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(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
25 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/520,385  
(B) FILING DATE:  
30 (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Goodman, Kenneth D.  
(B) REGISTRATION NUMBER: 30,460  
35 (C) REFERENCE/DOCKET NUMBER: UTFC433P--
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 713/787-1460  
(B) TELEFAX: 713/789-2679  
40
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:  
45 (A) LENGTH: 16 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10 GAAGGGCTTC TCGCTC  
16

(2) INFORMATION FOR SEQ ID NO:2:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

20

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

25

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

30 CTGAAGGGCT TCTTCC  
16

(2) INFORMATION FOR SEQ ID NO:3:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

40

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iv) ANTI-SENSE: YES

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGCTTTTGA ACTCTGCT  
18

**CLAIMS:**

1. A liposomal composition of antisense oligonucleotides, including (a) a liposome which consists essentially of neutral phospholipids, and (b) an antisense oligonucleotide that is entrapped in the liposome and is selected from the group  
5 consisting of phosphodiester oligonucleotides, phosphorothioate oligonucleotides, and p-ethoxy oligonucleotides.
2. The composition of claim 1, where the phospholipids are phosphatidylcholines.
3. The composition of claim 1, where the phospholipids are  
10 dioleoylphosphatidyl choline.
4. The composition of claim 1, where the antisense oligonucleotide is a phosphodiester oligonucleotide, and the molar ratio of phospholipid to oligo is less than about 3,000:1.
5. The composition of claim 1, where the antisense oligonucleotide is a  
15 phosphorothioate oligonucleotide, and the molar ratio of phospholipid to oligo is between about 10:1 and about 50:1.
6. The composition of claim 1, where the antisense oligonucleotide is a p-ethoxy oligonucleotide, and the molar ratio of phospholipid to oligo is between about 5:1 and about 100:1.
- 20 7. The composition of claim 1, where the antisense oligonucleotide is a p-ethoxy oligonucleotide which consists essentially of a nucleic acid molecule having the sequence GAAGGGCTTCTGCGTC.

8. The composition of claim 1, where the antisense oligonucleotide is a p-ethoxy oligonucleotide which consists essentially of a nucleic acid molecule having the sequence CTGAAGGGCTTCTTCC.

9. The composition of claim 1, where the antisense oligonucleotide is a p-ethoxy oligonucleotide which consists essentially of a nucleic acid molecule having the sequence GGGCTTTTGAAGCTCTGCT.

10. A method of inhibiting the growth of tumor cells, including the step of administering to a mammalian subject having a tumor an amount effective to inhibit the growth of tumor cells of a composition that includes (a) a liposome which consists essentially of neutral phospholipids, and (b) an antisense oligonucleotide that is entrapped in the liposome and is selected from the group consisting of phosphodiester oligonucleotides, phosphorothioate oligonucleotides, and p-ethoxy oligonucleotides.

11. A method of preparing a liposomal composition of antisense oligonucleotides, including the steps of:

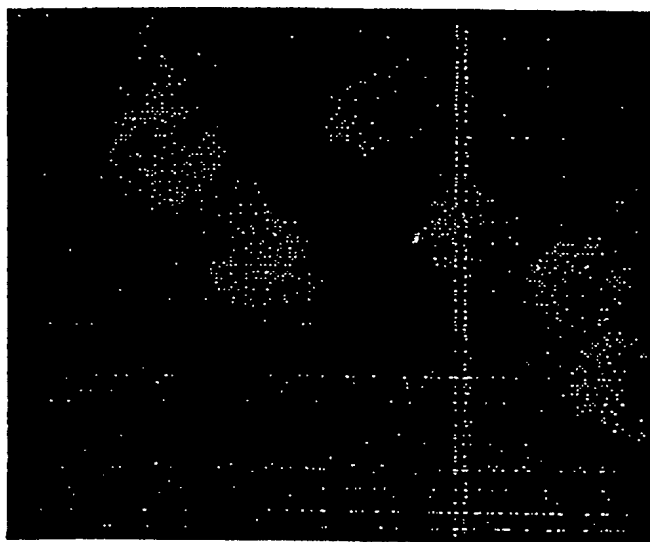
(a) hydrating a lyophilized composition that consists essentially of neutral phospholipids and an antisense oligonucleotide that is selected from the group consisting of phosphodiester oligonucleotides, phosphorothioate oligonucleotides, and p-ethoxy oligonucleotides, thereby forming an aqueous suspension which includes free oligonucleotide and liposomes entrapping oligonucleotide; and

(b) separating the free oligonucleotide from the liposomes by dialysis.

12. The method of claim 11, where the aqueous suspension is sonicated before dialysis.

Fig. 1

1A



1B

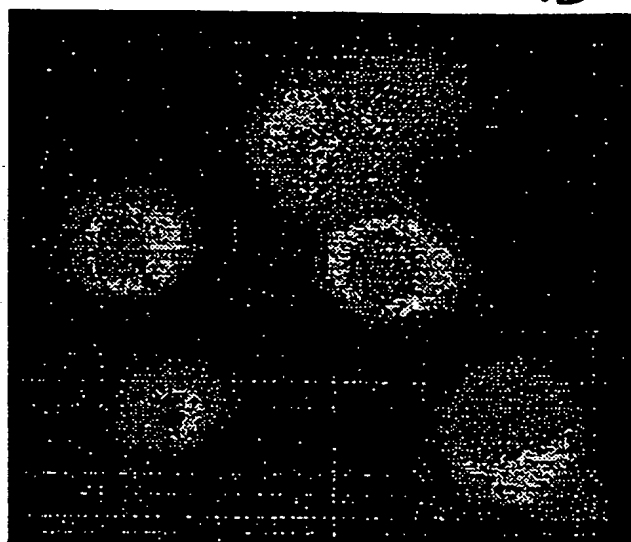


Fig. 2A

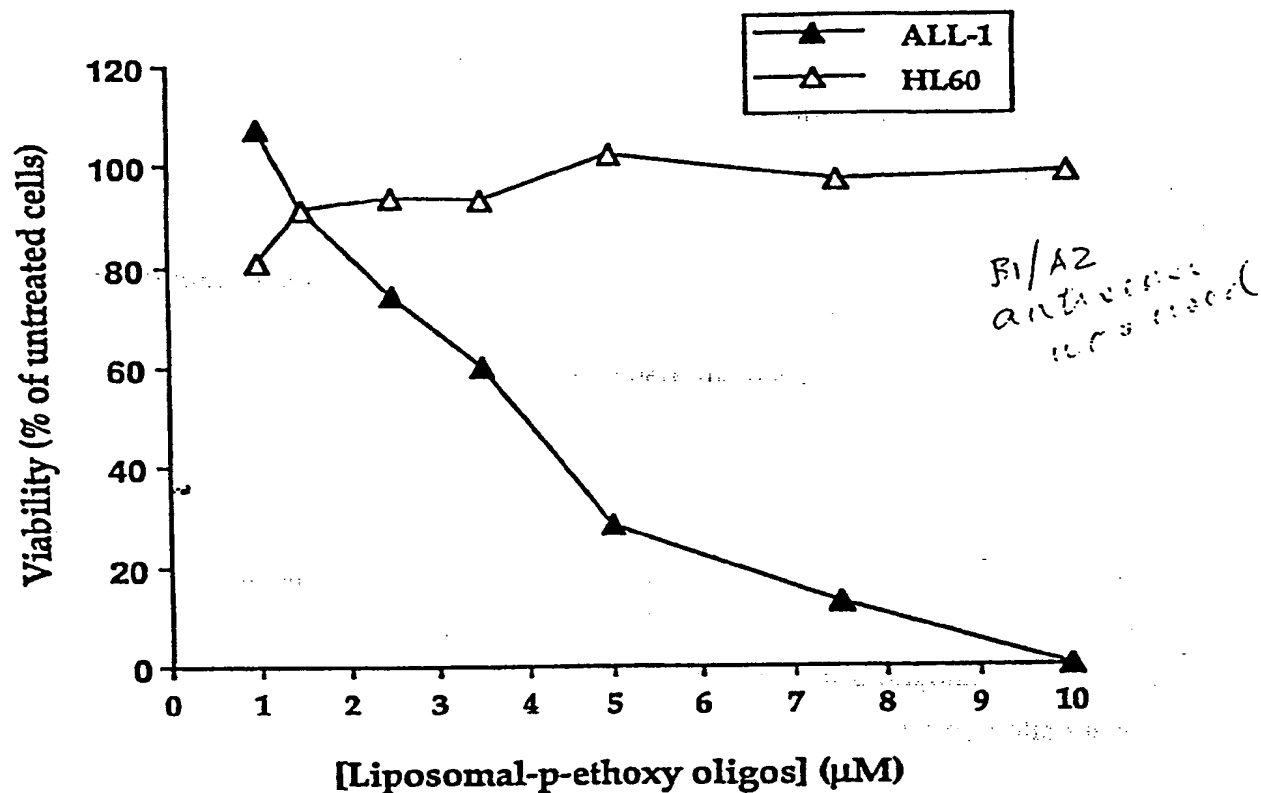


Fig. 2B

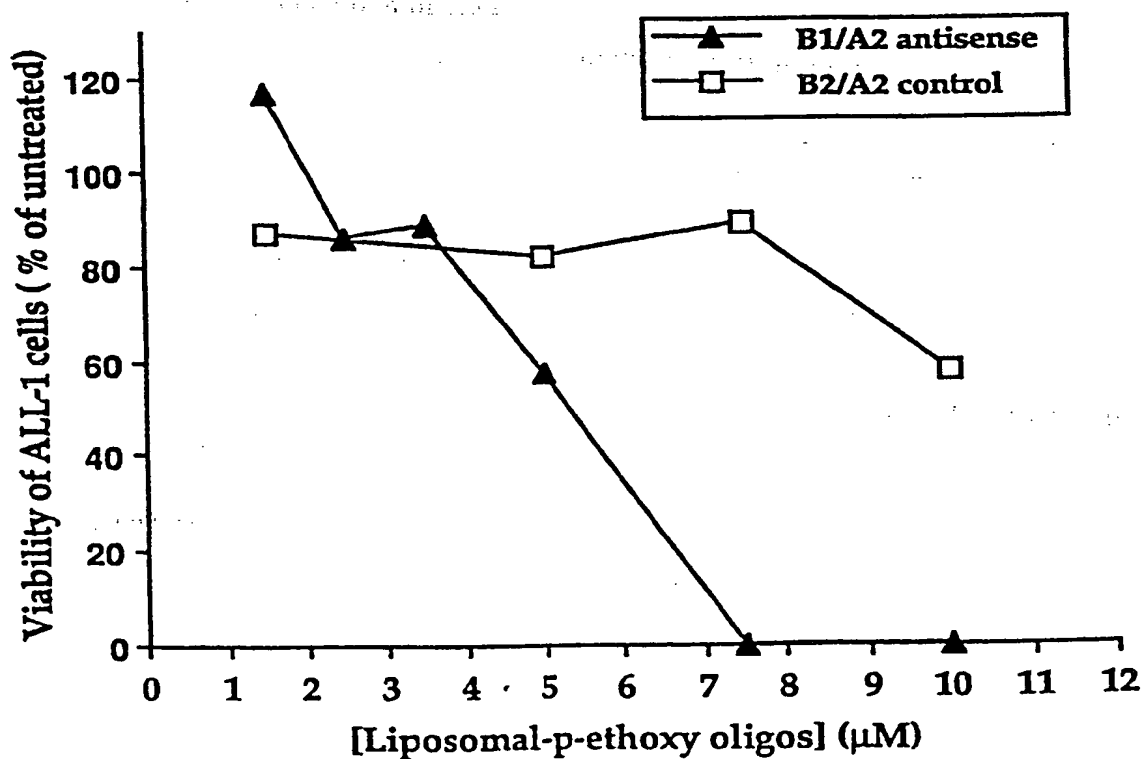
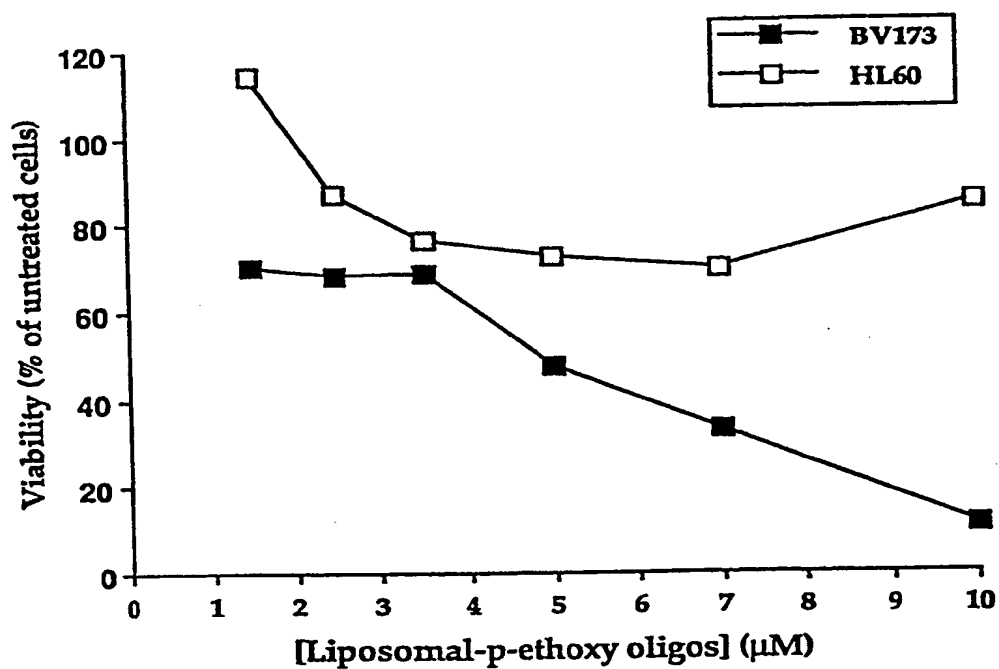




Fig. 3A



(B2/A2  
antisense  
was 1.0)

Fig. 3B

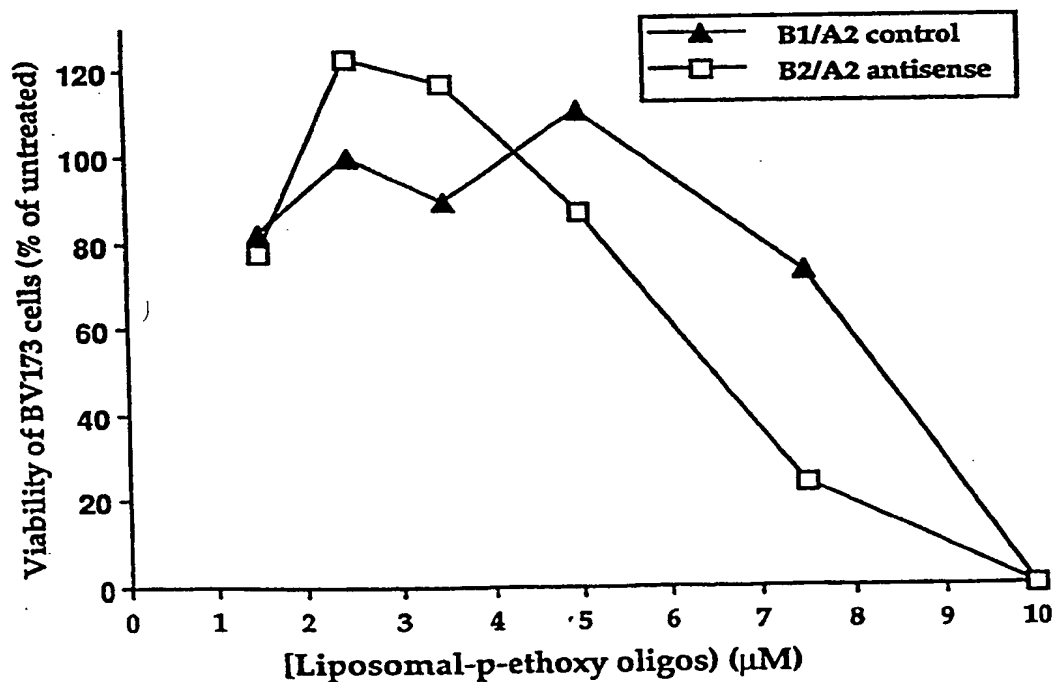


Fig. 4A

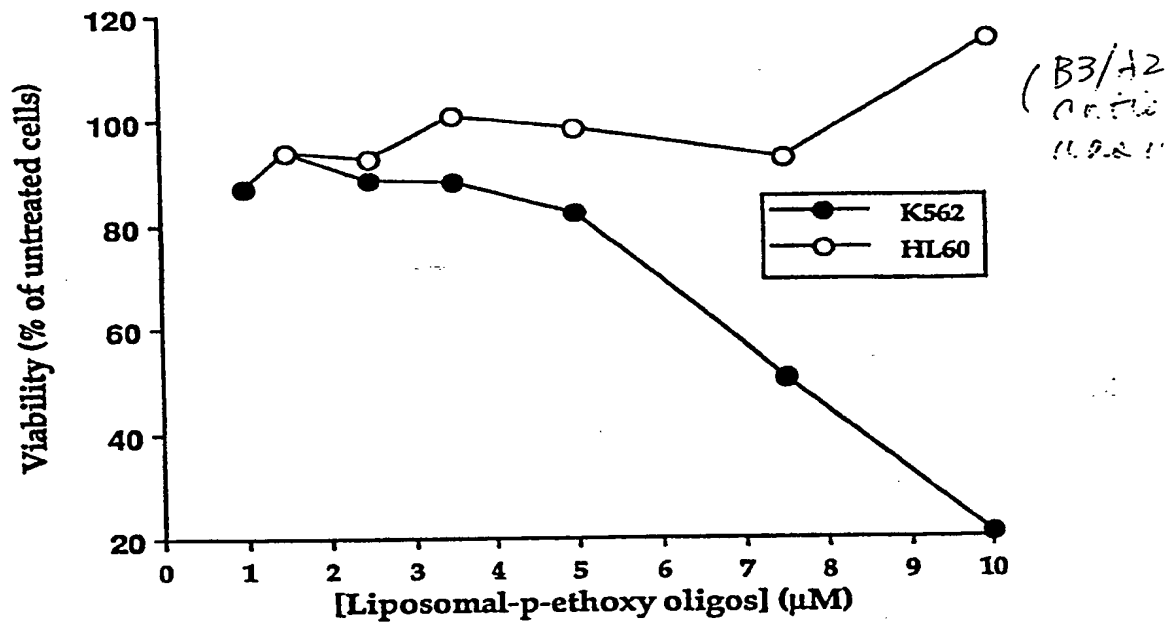


Fig. 4B

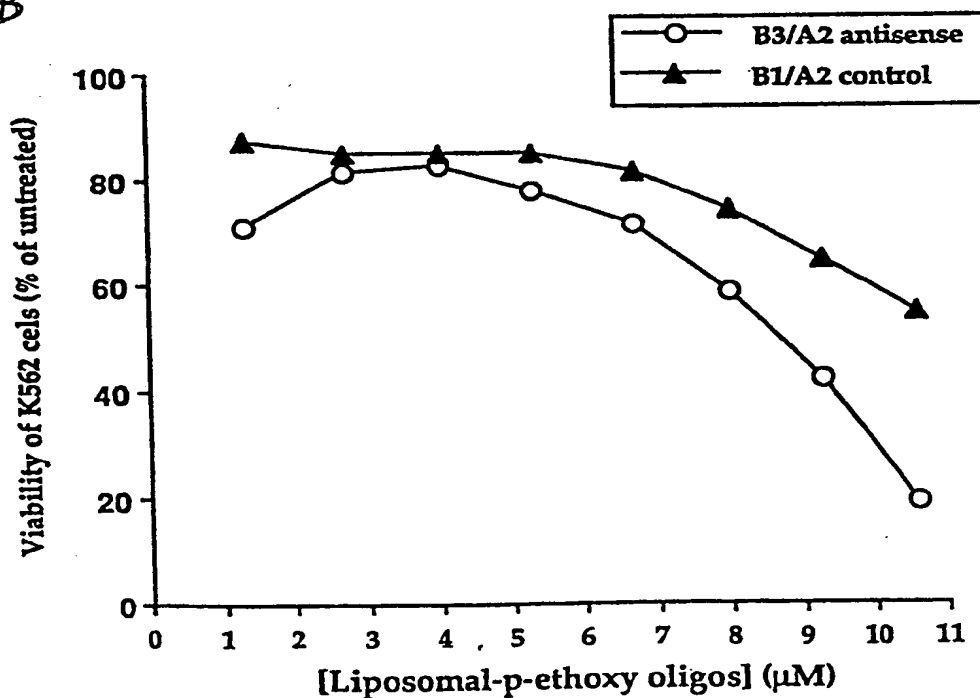
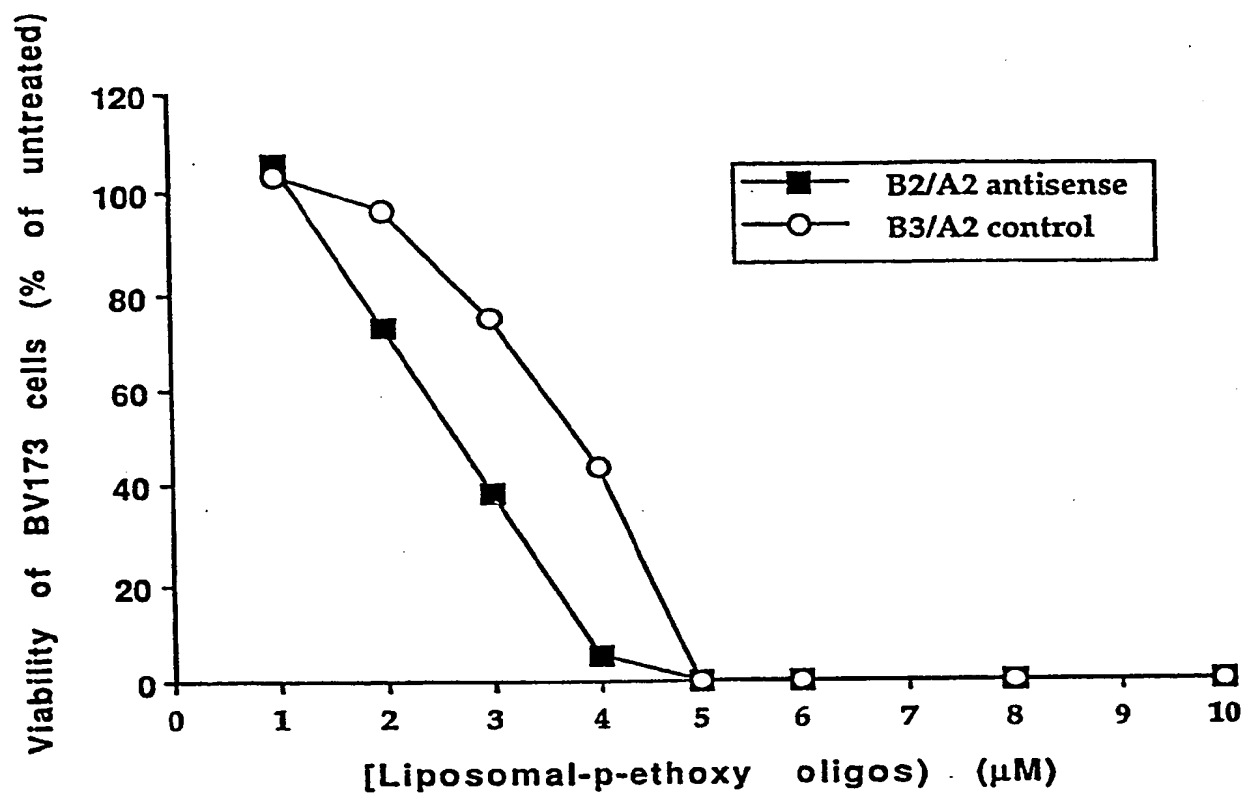


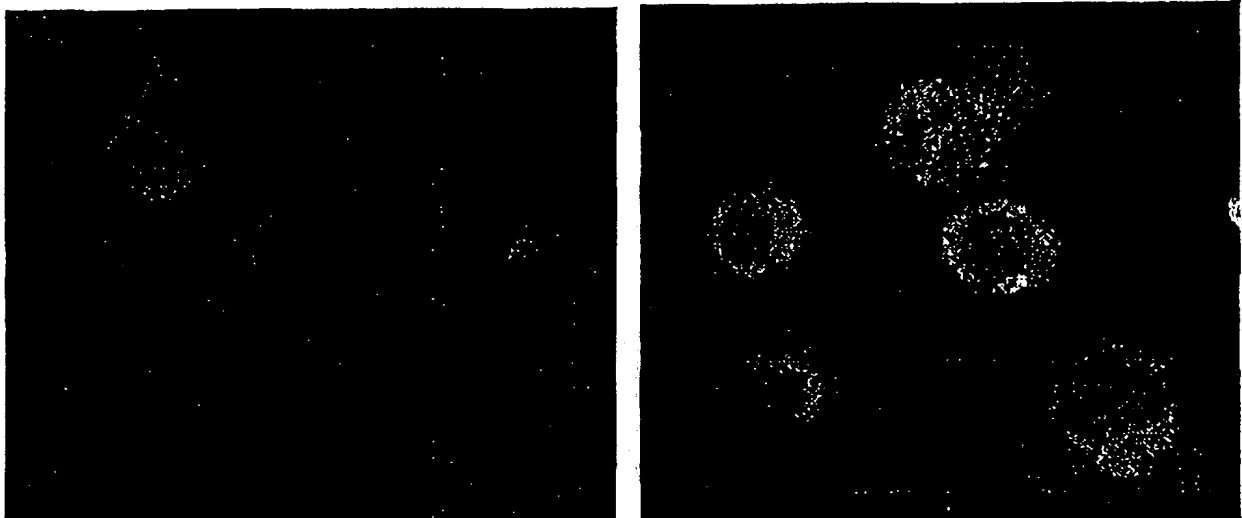
Fig. 5



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 9/127, C12N 15/11</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 97/07784</b> <b>(43) International Publication Date:</b> 6 March 1997 (06.03.97)
<b>(21) International Application Number:</b> PCT/US96/14146 <b>(22) International Filing Date:</b> 26 August 1996 (26.08.96) <b>(30) Priority Data:</b> 08/520,385 29 August 1995 (29.08.95) US <b>(71) Applicant:</b> BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US). <b>(72) Inventors:</b> LOPEZ-BERESTEIN, Gabriel; 5630 Rutherglenn, Houston, TX 77096 (US). TARI, Ana, Maria; 16 Charleston Park Drive #1703, Houston, TX 77025 (US). <b>(74) Agent:</b> GOODMAN, Kenneth, D.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).	<b>(81) Designated States:</b> AL, AM, AT, AU, BB, BG, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> <b>(88) Date of publication of the International search report:</b> 24 April 1997 (24.04.97)	
<b>(54) Title:</b> LIPOSOMAL PHOSPHODIESTER, PHOSPHOROTHIOATE, AND p-ETHOXY OLIGONUCLEOTIDES		
		
<b>(57) Abstract</b>  An improved delivery system for antisense oligonucleotides involves a liposomal composition, comprising a liposome which consists essentially of neutral phospholipids and an antisense oligonucleotide that is entrapped in the liposome and is selected from the group consisting of phosphodiester oligonucleotides, phosphorothioate oligonucleotides, and p-ethoxy oligonucleotides.		

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/14146

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K9/127 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 41 10 085 A (BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 1 October 1992 see page 2, line 65 - page 3, line 20 see page 23, line 37 - line 48 ---	1,2
Y	WO 95 03788 A (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 9 February 1995 see page 8, line 8 - page 11, line 31 --- -/--	1-4,6, 10-12

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*&\* document member of the same patent family

Date of the actual completion of the international search

10 March 1997

Date of mailing of the international search report

14.03.97

Name and mailing address of the ISA

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NL - 2280 HV Rijswijk  
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Authorized officer

Benz, K

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/14146

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>NUCLEIC ACIDS RESEARCH, vol. 19, no. 20, 25 October 1991, EYNSHAM , OXFORD (GB), pages 5551-5559, XP002027215 S. AKHTAR ET AL.: "interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes) " see the whole document see page 5553; table 1 ---</p>	<p>1-4,6, 10-12</p>
Y	<p>SCIENCE, vol. 261, no. 5124, 20 August 1993, WASHINGTON (US), pages 1004-1012, XP002027216 C.A. STEIN ET AL. : "antisense oligonucleotides as therapeutic agents-is the bullet really magical" see page 1005, column 1; figure 1 -----</p>	<p>1-4,6, 10-12</p>



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/14146

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 10  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
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restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/14146

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4110085 A	01-10-92	NONE	
WO 9503788 A	09-02-95	US 5417978 A AU 7407994 A CA 2168243 A EP 0711149 A JP 9501160 T	23-05-95 28-02-95 09-02-95 15-05-96 04-02-97

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